

1     Stem Cells

2

3     The present invention relates to the culture of  
4     primate embryonic stem cells, to the provision of  
5     feeder cells of human origin to support embryonic  
6     stem cell culture, and to the provision of  
7     fibroblast cells for therapeutic use.

8

9     Embryonic stem cells are undifferentiated cells  
10    able to proliferate for long periods and which can  
11    be induced to differentiate into any type of adult  
12    cell.

13

14    Human embryonic stem (hES) cells represent a great  
15    potential source of various cell types for  
16    therapeutic uses, pharmokinetic screening and  
17    functional genomics applications (Odorico et al.,  
18    2001, Stem Cells 19:193-204; Schuldiner et al.,  
19    2001, Brain Res 913:201-205; Zhang et al., 2002,  
20    Nat Biotechnol 19:1129-1133; He et al., 2003, Circ  
21    Res 93:32-39).

22

1 Typically embryonic stem cells are obtained from an  
2 embryo at the blastocyst stage (5 to 7 days), by  
3 extraction of the inner cell mass (ICM). The ICM  
4 is a group of approximately 30 cells located at one  
5 end of the internal cavity of the blastocyst.  
6 Pluripotent hES cell lines have been obtained from  
7 the ICM of Day 5 to 7 blastocysts (Thomson et al.,  
8 1998, Science 282:1145-1147; Reubinoff  
9 et al., 2000 Nature Biotechnol 18:399-404; Richards  
10 et al., 2002, Nature Biotechnol 20:933-936; Hovatta  
11 et al., 2003, Hum Reprod 18:1404-1409; Mitalipova  
12 et al., 2003, Stem Cells 21:521-526) but to date  
13 there have been no reports of obtaining hES cells  
14 from older blastocysts due to the difficulty of  
15 maintaining the viability of the blastocysts *in*  
16 *vitro*.

17  
18 Continuous culture of embryonic stem cells in an  
19 undifferentiated (pluripotent) state requires the  
20 presence of feeder layers such as mouse embryonic  
21 fibroblast (MEF) cells (Thomson et al., 1998,  
22 Science 282:1145-1147; Reubinoff et al., 2000, Nat  
23 Biotechnol 18:399-404), STO cells (Park et al.,  
24 2003, Bio Reprod 69:2007-2017), human foreskin  
25 fibroblasts (Hovatta et al., 2003, Hum Reprod  
26 18:1404-14069) human adult fallopian tubal  
27 epithelial cells, human fetal muscle and human  
28 fetal skin cells (Richards et al. 2002, Nature  
29 Biotechnol 20:933-935), or adult skin fibroblast  
30 cell lines (Richards et al. 2003, Stem Cells  
31 21:546-556). Alternatively, the culture media can  
32 be conditioned by growing the feeder cells in the

1 medium and then harvesting the medium for  
2 subsequent stem cell culture (see WO-A-99/20741).  
3 Whilst this method is referred to as "feeder-free"  
4 culture, nonetheless there is still a reliance on  
5 the feeder cells to culture isolated ICMs and to  
6 condition the media and hence there is potential  
7 for pathogen transmission.

8  
9 Unfortunately the use of feeder cells for the  
10 culture of hES cells limits their medical  
11 application for several reasons: xenogeneic and  
12 allogeneic feeder cells bear the risk of  
13 transmitting pathogens and other unidentified risk  
14 factors (Richards et al., 2002, Nat Biotechnol  
15 20:933-936; Hovatta et al., 2003, Hum Reprod  
16 18:1404-1409). Also, not all human feeder cells  
17 and cell-free matrices support the culture of hES  
18 cells equally well (Richards et al., 2002, Nat  
19 Biotechnol 20:933-936; Richards et al., 2003, Stem  
20 Cells 21:546-556), and the availability of human  
21 cells from aborted fetuses or Fallopian tubes is  
22 relatively low. Additionally there are ethical  
23 concerns regarding the derivation of feeder cells  
24 from aborted human fetuses.

25  
26 For example, WO-A-03/78611 describes a method of  
27 culturing human fibroblasts delivered from aborted  
28 human fetuses, typically of 4 to 6 week gestation.  
29 The fibroblasts are cultured from the rib region of  
30 the embryo and are described as being suitable to  
31 support human embryonic stem cell culture. However

1     this method relies upon the donation of aborted  
2     foetuses to maintain a supply of fibroblasts.  
3     US-A-2002/0072117 and US 6,642,048 describe the  
4     production of a human embryonic stem cell line by  
5     culturing the ICM of blastocysts and subsequently  
6     inducing the embryonic stem cells to form embryoid  
7     bodies and to differentiate into mixed  
8     differentiated cell populations. Cells having a  
9     morphology typical of fibroblasts were selected for  
10    use as feeder layers or to condition cell culture  
11    media for feeder-free culture. However no markers  
12    typical of fibroblasts were noted as being present  
13    on these cells.

14

15    There remains a need to culture primate embryonic  
16    stem (pES) cells, especially hES cells intended for  
17    therapeutic use, using only feeder cells of the  
18    same species or media conditioned by such feeder  
19    cells, to reduce the risk of cross-species pathogen  
20    transmission. Additionally, as mentioned above,  
21    the use of aborted foetuses as a source of human  
22    feeder cells is recognised to be of ethical concern  
23    and an alternative source of suitable feeder cells  
24    is required.

25

26    The present invention provides a novel human  
27    embryonic stem (hES) cell line. The novel cell  
28    line is termed hES-NCL1. A sample of the hES-NCL1  
29    cell line was deposited in accordance with the  
30    Budapest Treaty on 13 January 2005 at the National  
31    Institute for Biological Standards and Control  
32    (NIBSC), Blanche Lane, South Mimms, Potters Bar

1 Herts., EN6 3QC. The Accession Number allocated to  
2 the deposit was P-05-001.

3  
4 The hES cell line described above was isolated  
5 using novel methodology, which forms a further  
6 aspect of this invention, and was noted to  
7 spontaneously differentiate into fibroblast-like  
8 cells in the absence of any trigger and without the  
9 formation of embryoid bodies. The fibroblast-like  
10 cells so formed expressed the specific fibroblast  
11 marker AFSP (anti-fibroblast cell surface specific  
12 protein, from Sigma). A photomicrograph of the  
13 stained fibroblast-like cells is shown at Figures  
14 2B, C, D. The stem cell derived fibroblast-like  
15 cells, their formation and their use in culture (as  
16 feeder cells or to condition the culture media) of  
17 animal embryos (including non-human embryos such as  
18 non-human primate embryos as well as human embryos)  
19 or embryonic or non-embryonic stem cells (which  
20 embryonic or non-embryonic stem cells may be of  
21 human or non-human origin), and in therapy forms a  
22 further aspect of the present invention and is  
23 discussed further below.

24  
25 In one aspect, the present invention provides a  
26 method of culturing a blastocyst, said method  
27 comprising exposing said blastocyst to Buffalo rat  
28 liver cells or media conditioned thereby for at  
29 least 12 hours.

30

1 The Buffalo rat liver cells may conveniently be  
2 present in the cell culture media or, more  
3 preferably, will be used to condition that media.

4

5 The blastocyst may be exposed to the Buffalo rat  
6 liver cells or media conditioned thereby for a  
7 minimum period of 24 hours, 36 hours, 48 hours, 60  
8 hours or 72 hours. We have found that an exposure  
9 period of approximately 2 days is sufficient.

10 Where the blastocyst is to be used to generate  
11 pluripotent embryonic stem cells, it is desirably  
12 exposed to the Buffalo rat liver cells or media  
13 conditioned thereby in the period immediately prior  
14 to the extraction of cells of the ICM. Benefits  
15 may also be obtained from exposing the blastocyst  
16 to Buffalo rat liver cells or media conditioned  
17 thereby where the blastocyst is intended for  
18 implantation as part of IVF treatment.

19

20 In more detail, one protocol for culturing a  
21 blastocyst according to the present invention  
22 comprises:

- 23 i) culturing said blastocyst from fertilisation
- 24 in G1 media;
- 25 ii) transferring said blastocyst of step i) to
- 26 G2.3 media and maintaining said blastocyst in
- 27 the G2.3 media; and
- 28 iii) transferring said blastocyst of step ii) to
- 29 cell culture media conditioned by Buffalo rat
- 30 liver cells.

31

1 The G1 and G2.3 media referred to above can be  
2 obtained from Vitrolife Sweden AB, Kungsbacka,  
3 Sweden.

4  
5 G-1<sup>TM</sup> is a media designed to support the  
6 development of embryos to the 8-cell stage, ie.  
7 from pro-cleavage to day 2 or 3. The media  
8 contains carbohydrates, amino acids and chelators,  
9 as well as Hyaluronan and is bicarbonate buffered.

10 In more detail, the G-1<sup>TM</sup> media contains:

11 Alanine	Penicillin G
12 Alanyl-glutamine	Potassium chloride
13 Asparagine	Proline
14 Aspartate	Serine
15 Calcium chloride	Sodium bicarbonate
16 EDTA	Sodium chloride
17 Glucose	Sodium dihydrogen phosphate
18 Glutamate	Sodium lactate
19 Glycine	Sodium pyruvate
20 Hyaluronan	Taurine
21 Magnesium sulphate	Water for injection (WFI)

22  
23 G-2<sup>TM</sup> is a cell culture media to support the  
24 development of embryos from around the 8-cell stage  
25 to the blastocyst stage. The media contains  
26 carbohydrates, amino acids and vitamins, as well as  
27 Hyaluronan, and is bicarbonate buffered. In more  
28 detail the G-2<sup>TM</sup> version 3 (ie. G2.3) media  
29 contains:

31 Alanine	Penicillin G
32 Alanyl-glutamine	Phenylalanine

8

1	Arginine	Potassium chloride
2	Asparagine	Proline
3	Aspartate	Pyridoxine
4	Calcium chloride	Riboflavin
5	Calcium pantothenate	Serine
6	Cystine	Sodium bicarbonate
7	Glucose	Sodium chloride
8	Glutamate	Sodium dihydrogen phosphate
9	Glycine	Sodium lactate
10	Histidine	Sodium pyruvate
11	Hyaluronan	Thiamine
12	Isoleucine	Threonine
13	Leucine	Tryptophan
14	Lysine	Tyrosine
15	Magnesium sulphate	Valine
16	Methionine	Water for injection (WFI)

17

18 The duration of step i) above may typically be from  
19 Day 0 (at fertilisation) to Day 3.

20

21 The duration of step ii) above may typically be for  
22 2 or 3 days, that is from Day 3 to Day 5 or 6.

23

24 The duration of step iii) above is for a minimum  
25 period of 24 hours as described above, but may  
26 typically be for 1 to 3 days.

27

28 In step iii) a preferred cell culture media  
29 consists of Dulbecco's modified Eagle's medium  
30 (DMEM, Invitrogen, Paisley, Scotland), optionally  
31 supplemented with 15% (v/v) Glasgow medium, and  
32 conditioned by Buffalo rat liver cells (see



1 Stojkovic et al., 1995, Biol Reprod 53:1500-1507).  
2 Typically conditioning by the Buffalo rat liver  
3 cells comprises culturing approximately 75000  
4 Buffalo rat liver cells/cm<sup>2</sup> in Glasgow medium for  
5 24-36 hours. The media is then recovered and  
6 frozen at -20°C until required.

7  
8 Using a blastocyst cultured as described above, the  
9 ICM can be extracted using routine techniques as  
10 late as Day 8, typically by immunosurgery (see  
11 Reubinoff et al., 2001, Hum Reprod 10:2187-2194).  
12 Blastocysts are cultured for 30 minutes in whole  
13 human antiserum (Sigma) diluted 1:5 in DMEM+FCS  
14 medium (i.e. 80% Dulbeco's modified Eagle's medium  
15 with 10-20% (v/v) fetal calf serum). Furthermore,  
16 the blastocysts are washed three times and cultured  
17 for another period of approximately 20 minutes in  
18 guinea pig complement (1:5). The isolated ICMs can  
19 be used for embryonic stem cell culture but could  
20 alternatively be implanted into a receptive female  
21 as part of an IVF treatment.

22  
23 For human blastocysts, the blastocyst will have  
24 been donated, with informed consent, as being  
25 superfluous to IVF treatment. For other (ie. non-  
26 human) primates, the ovulation cycle can be  
27 controlled by intramuscular injection of  
28 prostaglandin or a prostaglandin analogue, and the  
29 embryos harvested by a non-surgical uterine flush  
30 procedure (see Thompson et al., 1994, J Med  
31 Primatol 23:333-336) at day 8 following ovulation.

32

1 If the blastocyst is unhatched, the zona pellucida  
2 is removed by brief exposure to pronase. This step  
3 is not required for hatched embryos. The  
4 blastocyst is exposed to antiserum for 30 minutes.  
5 The blastocyst is then washed three times in DMEM,  
6 and exposed to a 1:5 dilution of Guinea pig  
7 complement (Gibco) for 20 minutes. After two  
8 further washes in DMEM, lysed trophectoderm cells  
9 are removed from the ICM by pipette and the ICM  
10 plated out on a suitable feeder layer. Embryonic  
11 stem cell lines are identified from the cultured  
12 ICM cells.

13

14 As mentioned above, the novel methodology enables  
15 the blastocyst to be cultured at a relatively late  
16 stage, day 8. At day 8 the number of cells  
17 obtainable from the ICM is considerably increased,  
18 but surprisingly these cells retain their  
19 pluripotent ability.

20

21 The present invention therefore provides a method  
22 of producing an embryonic stem cell line, said  
23 method comprising:

- 24 i) culturing a blastocyst as described above; and  
25 ii) extracting cells of the ICM from said  
26 blastocyst and culturing the cells to produce  
27 an embryonic stem cell line therefrom.

28

29 The reference to culturing the cells of the ICM  
30 extracted from the blastocyst in step ii) above  
31 includes the published protocols available and is

1 not especially dependent upon any particular  
2 culture conditions.

3  
4 The method of producing stem cells according to the  
5 present invention provides a generic and efficient  
6 method for the production of primate embryonic stem  
7 (pES) cell lines. The pES cell lines may be human  
8 embryonic stem (hES) cell lines. An exemplary hES  
9 cell line produced by this methodology is the cell  
10 line hES-NCL deposited as cell line P-05-001.

11 Alternatively the pES cells may be of non-human  
12 origin. The stem cell lines so produced are  
13 preferably of clinical and/or GMP grade.

14  
15 In one embodiment the stem cells of the present  
16 invention and/or obtained by the method described  
17 above are pluripotent stem cells.

18  
19 In one embodiment the stem cells of the present  
20 invention and/or obtained by the method described  
21 above are multipotent stem cells.

22  
23 In one embodiment the stem cells of the present  
24 invention and/or obtained by the method described  
25 above are unipotent stem cells.

26  
27 One suitable medium for the isolation of embryonic  
28 stem cells consists of 80% Dulbecco's modified  
29 Eagle's medium (DMEM; obtainable from Invitrogen or  
30 Gibco) with 10-20% (v/v) fetal calf serum (FCS,  
31 Hyclone, Logan, UT). Optionally the medium may  
32 also include one or more of 0.1 mM  $\beta$ -

12

1 mercaptoethanol (Sigma), up to 1% (v/v) non-  
2 essential amino acid stock (Gibco), 1% (v/v)  
3 antibiotic, such as penicillin-streptomycin  
4 (Invitrogen), and/or 4ng/ml bFGF (Invitrogen). To  
5 date details of several specific media suitable for  
6 embryonic stem cell culture have been published in  
7 the literature - see for example Thomson et al.,  
8 1998, Science 282:1145-1147; Xu et al., 2001,  
9 Nature Biotechnol 19:971-974; Richards et al.,  
10 2002, Nature Biotechnol 20:933-936; and Richards et  
11 al., 2003, Stem Cells 21:546-556.

12

13 Feeder cells which may be used for stem cell  
14 culture include mouse embryonic stem cells (MEF),  
15 STO cells, foetal muscle, skin and foreskin cells,  
16 adult Fallopian tube epithelial cells (Richards et  
17 al., 2002, Nat Biotechnol 20:933-936; Amit et al.,  
18 2003, Biol Reprod 68:2150-2156; Hovatta et al.,  
19 2003, Hum Reprod 18:1404-1409; Park et al., 2003,  
20 Biol Reprod 69, 2007-2014; Richards et al., 2003,  
21 Stem Cells 21:546-556), adult bone marrow cells  
22 (Cheng et al., 2003, Stem Cells 21:131-142), or on  
23 coated dishes with animal based ingredients with  
24 the addition of MEF cell conditioned media (Xu et  
25 al., 2001, Nature Biotechnol 19:971-974).

26

27 The method of culturing a blastocyst and the method  
28 of producing embryonic stem cell lines as described  
29 above are both suitable for use with blastocysts of  
30 primate origin, including blastocysts of human or  
31 non-human origin.

32

1 The human embryonic stem cells of the present  
2 invention are characterised by at least one of the  
3 following;  
4 i) presence of the cell surface markers TRA-1-60,  
5 GTCM2, and SSEA-4;  
6 ii) expression of *Oct-4*;  
7 iii) expression of *NANOG*;  
8 iv) expression of *REX-1*; and/or  
9 v) expression of *TERT*.

10

11 In one embodiment at least 2 or more of the  
12 characteristics listed above are present,  
13 preferably 3 or more of the characteristics are  
14 present, especially 4 or more, more preferably all  
15 of the above characteristics are present in the  
16 stem cells.

17

18 The antigen SSEA-4 is a glycolipid cell marker.  
19 Specific antibodies to identify this marker are  
20 available from the Development Studies Hybridoma  
21 Bank, DSHB, Iowa City, IA.

22

23 The cell surface marker TRA-1-60 is recognised by  
24 antibodies produced by hybridomas developed by  
25 Peter Andrews of the University of Sheffield (see  
26 Andrews et al., "Cell lines from human germ cell  
27 tumours" pages 207-246 in *Teratocarcinomas and  
28 Embryonic Stem Cells: A Practical Approach*, Ed.  
29 Robertson, Oxford, 1987). TRA1-60 is also  
30 commercially available (Chemicon). Both GTCM2 and  
31 TG343 are described in Cooper et al., 2002, J.  
32 Anat. 200(Pt 3):259-65.

1 The embryonic stem cell line according to the  
2 present invention as described above or which is  
3 produced according to the method of the present  
4 invention as described above (and specifically the  
5 stem cell line hES-NCL1) can be used for screening  
6 and/or to produce differentiated cells of specific  
7 cell types for therapeutic purposes (e.g. for  
8 implantation to replace damaged, diseased or  
9 missing tissue). The stem cell lines (e.g. hES-  
10 NCL1) can be used to screen agents (e.g. chemical  
11 compounds or compositions) for toxicity and/or for  
12 therapeutic efficacy (i.e. pharmacological  
13 activity).

14

15 In a further aspect, the present invention provides  
16 a method of screening an agent for toxicity and/or  
17 for therapeutic efficacy, said method comprising:

- 18 a) exposing an embryonic stem cell line  
19 according to the present invention (e.g.  
20 hES-NCL1) or obtained by the method  
21 described above to said agent;  
22 b) monitoring any alteration in viability  
23 and/or metabolism of said stem cells; and  
24 c) determining any toxic or therapeutic effect  
25 of said agent.

26

27 Additionally, the method of producing a stem cell  
28 line according to the present invention as  
29 described above, and the stem cell lines produced  
30 thereby (e.g. hES-NCL1) may be used in the creation  
31 of an embryonic stem cell bank for use in screening  
32 and/or to produce differentiated cells of specific

1 cell types for therapeutic purposes. The stem cell  
2 bank, which forms a further aspect of the present  
3 invention, will consist of a multiplicity of  
4 genetically distinct stem cell lines. The stem  
5 cell lines forming the stem cell bank will usually  
6 be of primate embryonic stem cells such as human  
7 embryonic stem cells or non-human embryonic stem  
8 cells. The embryonic stem cell bank can be used to  
9 screen agents (e.g. chemical compounds or  
10 compositions) for toxicity and/or for therapeutic  
11 efficacy (i.e. pharmacological activity).  
12

13 Thus, in a yet further aspect, the present  
14 invention provides a method of screening an agent  
15 for toxicity and/or for therapeutic efficacy, said  
16 method comprising:

- 17 a) exposing an embryonic stem cell bank  
18 comprising a multiplicity of embryonic stem  
19 cell lines according to the present invention  
20 or obtained by the method described above to  
21 said agent;
  - 22 b) monitoring any alteration in viability and/or  
23 metabolism of said stem cells; and
  - 24 c) determining any toxic or therapeutic effect of  
25 said agent.
- 26

27 As briefly mentioned above, it was noted that the  
28 embryonic stem cell line established from a  
29 blastocyst cultured as described above according to  
30 the present invention spontaneously differentiated  
31 into fibroblast-like cells without formation of  
32 embryoid bodies. Such spontaneous differentiation

1 into a single cell type was totally unexpected.  
2 These fibroblast-like cells then acted as a feeder  
3 layer for the remaining undifferentiated embryonic  
4 stem cells of the culture. The stem cell derived  
5 fibroblast-like cells and the embryonic stem cells  
6 supported thereby were autogeneic.

7  
8 The spontaneous differentiation of hES cells in a  
9 feeder-free culture into a mixture of cell types,  
10 including fibroblast-like cells, has already been  
11 described (see Park et al., 2003, Biol Reprod  
12 69:2007-2014) but in that study the differentiation  
13 was observed in the centre of the hES cell  
14 colonies. This differs to the present invention  
15 where differentiation occurs at the periphery of  
16 the colony. Moreover in the present invention only  
17 fibroblast-like cells were observed and no other  
18 cell types were noted to be present.

19  
20 In one embodiment the present invention provides a  
21 method of producing fibroblast-like cells, said  
22 method comprising:

- 23 i. providing a stem cell line according to  
24 the present invention; and  
25 ii. allowing cells of said stem cell line to  
26 differentiate into stem cell derived  
27 fibroblast-like cells.

28  
29 In an alternative embodiment the present invention  
30 provides a method of producing fibroblast-like  
31 cells, said method comprising:

- 32 i) culturing a blastocyst as described above;



- 1     ii) extracting cells of the ICM from said  
2         blastocyst and culturing the cells to produce  
3         an embryonic stem cell line therefrom; and  
4     iii) allowing cells of said embryonic stem cell  
5         line to differentiate into stem cell derived  
6         fibroblast-like cells.

7  
8     The stem cell derived fibroblast-like cells are  
9     produced without requiring a specific stimulant,  
10    e.g. growth factor or change in physical growth  
11    conditions (e.g. allowing the cells to become  
12    crowded).

13  
14    One suitable method for obtaining differentiation  
15    of the stem cells into fibroblast-like cells was  
16    simply to transfer the stem cells to cell culture  
17    media in the absence of feeder cells or feeder cell  
18    conditioning. The stem cells responded by  
19    differentiation of a proportion of the stem cells  
20    which then acted as feeder cells for the non-  
21    differentiated remaining stem cells. Thus  
22    obtaining differentiation into fibroblast-like  
23    cells was possible using an extremely easy one-step  
24    process, avoiding the need for time-consuming  
25    procedures and allowing the differentiation to be  
26    fully controlled under *in vitro* conditions.

27  
28    The stem cell derived fibroblast-like cells are  
29    characterised by a morphology typical of the cell  
30    type, ie. long flat cells with an elongated,  
31    condensed nucleus. The cytoplasmic processes

1       therein resemble those found in fibroblasts of  
2       connective tissue.

3  
4       The fibroblast-like cells of the present invention  
5       are positive for the cell surface marker AFSP. In  
6       addition, the identity of hES cells-derived  
7       fibroblasts was confirmed by karyotyping and DNA  
8       analysis of both stem cells and hES cells-derived  
9       fibroblasts. This confirmed that hES cells-derived  
10      fibroblasts are autogeneic i.e. of the same origin  
11      as the stem cells.

12  
13     The fibroblast-like cells according to the present  
14     invention could be easily immortalised using known  
15     techniques to provide a long term source of the  
16     cells.

17  
18     The present invention also provides a novel human  
19     embryonic stem cell derived fibroblast-like cell  
20     line. The novel fibroblast-like cell line, termed  
21     hESCdF-NCL, has been deposited at the European  
22     Collection of Cell Cultures (ECACC) on 19 January  
23     2004 under Accession No 04010601.

24  
25     The fibroblast-like cells and media conditioned by  
26     the fibroblast-like cells of the present invention  
27     are suitable to support the growth of embryos. The  
28     fibroblast-like cells and media conditioned by the  
29     fibroblast-like cells of the present invention are  
30     alternatively suitable to support the growth of  
31     stem cells, especially non-human primate embryonic  
32     stem cells or human embryonic stem cells. Other

1 types of stem cells needing the use of feeder cells  
2 to survive are also included and particular mention  
3 may be made of unipotential and pluripotential stem  
4 cells such as adult stem cells, haemopoietic stem  
5 cells, mesenchymal stem cells, osteogenic stem  
6 cells, chondrogenic stem cells, neuronal stem  
7 cells, gonadal stem cells, epidermal stem cells and  
8 somatic/progenitor stem cells. Where the  
9 fibroblast-like cells of the present invention are  
10 used to support human stem cells, the fibroblast-  
11 like cells are desirably autogeneic thereto but  
12 xenogeneic feeder cells may be used following  
13 screening to ensure that they are pathogen-free.

14

15 In a further aspect, the present invention provides  
16 a self-feeder system for the growth of  
17 undifferentiated stem cells, said system comprising

- 18 i) culturing a blastocyst as described above,  
19 extracting cells of the ICM from said  
20 blastocyst and culturing the cells to produce  
21 an embryonic stem cell line therefrom, or  
22 providing a stem cell line according to the  
23 present invention; and  
24 ii) allowing some of the cells of said embryonic  
25 stem cell line to differentiate into stem  
26 cell derived fibroblast-like cells whilst the  
27 remainder of the cells of said embryonic stem  
28 cell line remain in an undifferentiated  
29 pluripotent, multipotent or unipotent state,  
30 whereby said stem cell derived fibroblast-  
31 like cells act as autogeneic feeder cells for  
32 said stem cells.

1 The fibroblast-like cells may be used directly as  
2 feeder cells to support stem cell culture (eg are  
3 grown as a confluent surface in contact with the  
4 stem cells) or may be used to condition media for  
5 use in stem cell culture. Generally, where the  
6 media is to be conditioned, the fibroblast-like  
7 cells are grown in the media for a predetermined  
8 period of typically 24 hours, although periods of  
9 up to a maximum of 9 days may be used, before the  
10 media is removed and transferred to the stem cells.

11

12 There are several advantages for using hES cells  
13 derived fibroblasts as feeder cells: i) feeder  
14 derived from hES cells offers more secure  
15 autogeneic/genotypically homogenous system for  
16 prolonged growth of undifferentiated hES cells, ii)  
17 feeders differentiated from first clinical-grade  
18 hES cell line could be used worldwide as initial  
19 monolayer for growth of isolated ICMS to eliminate  
20 transfer of pathogens, iii) the long proliferation  
21 time of already derived hES cell lines allows  
22 screening for viral contamination, iv) medium  
23 conditioned by hESdF can be used for feeder-free  
24 growth of hES cells thus avoiding potential viral  
25 transfer from the MEF conditioned media used to  
26 date, v) due to the low bioburden, embryonic  
27 tissues perform better support *in vitro* than adult  
28 tissues (see Richards et al., 2003, Stem Cells  
29 21:546-556), vi) derivation and culture of hESdF is  
30 fully controlled and not time consuming, vii)  
31 derived feeder cells could be easily immortalized  
32 to provide a long-term source of this tissue, viii)

1     *in vitro* studies on cell-to-cell contacts and  
2     identification of isolated soluble factors could  
3     significantly improve cell-culture, cell-  
4     transplantation and tissueengineering avoiding at  
5     the same time expensive tissue-biopsy and  
6     unnecessary sacrifice of animals.

7  
8     Accordingly, the present invention further provides  
9     a method of culturing a primate embryonic stem cell  
10    line, such as a human embryonic stem cell line, to  
11    maintain the viability of eggs prior to or during  
12    fertilisation and/or to culture blastocysts or  
13    embryos intended for implantation into a receptive  
14    female to establish a pregnancy (i.e. as part of an  
15    IVF procedure). The method comprises providing  
16    fibroblast-like cells according to the present  
17    invention or obtained by the method described above  
18    as feeder cells or to condition the cell culture  
19    media. Advantageously the fibroblast-like cells  
20    selected will be obtained from an embryonic stem  
21    cell line of the same origin or species, and will  
22    be previously screened to ensure pathogen-free  
23    status. This approach enables the complete  
24    elimination of animal ingredients for the culture  
25    of undifferentiated hES cells and avoids the  
26    potential of viral transfer which may occur when  
27    MEF conditioned media or conditioned media from  
28    other feeders is used for stem cell culture.

29  
30    We have found that the use of the fibroblast-like  
31    cells obtained according to the present invention  
32    (e.g. hESCdF-NCL) as feeder cells or to condition

1 the culture media enables the undifferentiated  
2 culture of the embryonic stem cells. It is  
3 anticipated that a similar ability will be obtained  
4 using other stem cell types. This is highly  
5 significant for the long term maintenance of such  
6 cell lines and also has the advantage that the  
7 extended culture period possible for the  
8 undifferentiated stem cell line enables the cell  
9 line to be screened for any potential pathogen  
10 (e.g. viral contamination).

11

12 Alternatively, the fibroblast-like cells can be  
13 used for therapy, for example to assist  
14 regeneration of wounds requiring fibroblast  
15 presence.

16

17 The presence of fibroblast cells, without  
18 contamination of other cell types is of particular  
19 advantage in therapy. One example of the use of  
20 the fibroblasts according to the present invention  
21 is the generation of skin grafts for use in  
22 treating wounds (for example burns) or in cosmetic  
23 or regenerative surgery.

24

25 The present invention will now be further described  
26 with reference to the following examples and  
27 figures, in which:

28

29 **Figure 1.** Morphology of human blastocysts and hES  
30 cells. Day 6 blastocysts (A) and hatched Day 8  
31 blastocysts (B). Note the presence of very well  
32 organised inner cell mass in Day 8 blastocyst

recovered after three-step *in vitro* culture. Inner cell mass cells (C) grown on irradiated MEF 4 days after immunosurgery. Primary hES cells colony (D) grown on inactivated MEF cells. Same colony at high magnification (E). Bars: 50  $\mu\text{m}$  (A-D); 100  $\mu\text{m}$  (E).

**Figure 2.** Morphology and characterisation of hES cells-derived fibroblasts. Undifferentiated hES cells (A). Peripheric differentiation of hES cells into fibroblast-like cells in feeder-free conditions (B). Phase (C) and fluorescence (D) microscopy of hES cells-derived fibroblasts using AFSP antibody. Normal 46 + XX karyotypes of hES cells (E) and hES cells-derived fibroblasts (F). Microsatellite analysis of hES cells (G) and hES cells-derived fibroblasts (H). Bars: 50  $\mu\text{m}$  (A, C, D), 100  $\mu\text{m}$  (B).

**Figure 3.** Morphology of frozen/thawed hES-NCL1 colony cultured on frozen/thawed hES cell-derived fibroblasts. Bar: 50  $\mu\text{m}$ .

**Figure 4.** Morphology and characterisation of hES-NCL1 cells grown on  $\gamma$ -irradiated hESdF monolayer (A-F) or feeder-free (G, H). (A) Five days old vitrified hES-NCL1 colony cultured on frozen/thawed hESdF (passage 8). (B) Higher magnification of the same hES colony. Note typical morphology of hES cells i.e. small cells with prominent nucleoli. hES cells grown on hESdF stained with antibody recognising the TRA1-60 (D) and SSEA-4 (F) epitopes. hES cells grown on Matrigel (G) with

1 addition of hESdF conditioned medium stained with  
2 antibody recognising the GTCM2 epitope (H). Bars:  
3 200  $\mu$ m (A, E-H); 50  $\mu$ m (B); 100  $\mu$ m (C, D).  
4

5 **Figure 5.** Characterisation and karyotyping of hES-  
6 NCL1 cells grown on hESdF monolayer. RT-PCR  
7 analysis of undifferentiated hES cells grown on  
8 inactivated hESdF cells (A). PCR products obtained  
9 using primers specific for *OCT-4*, *NANOG*, *FOXD3*,  
10 *TERT*, *REX1* and *GAPDH*. HES cells (passage 31) grown  
11 on hESdF (passage 11) show normal female karyotype  
12 (46, XX) (B).  
13

14 **Figure 6.** Histological analysis of teratomas formed  
15 from grafted colonies of hES cells grown on  
16 inactivated hESdF in testis (A-C) and kidney (D-F)  
17 of SCID mice. (A) neural epithelium (ne); (B)  
18 aggregation of glandular cells with characteristic  
19 appearance of secretory acini (sa); (C) cartilage  
20 (cart); (D) wall of respiratory passage showing  
21 epithelium (ep), submucosa (sm), submucosal glands  
22 (sg). Epithelium contains occasional ciliated cells  
23 and numerous goblet cells secreting mucin (m); (E)  
24 Two types of epithelia: respiratory (top),  
25 keratinised skin (bottom). Submucosal glands (sg)  
26 located beneath pseudostratified ciliated (in  
27 parts) epithelium (ep). Structures of the skin  
28 include epidermis (ed), dermis (dm) and cornified  
29 layer (c). Note that the stratum granulosum (arrow)  
30 is characterised by intracellular granules which  
31 contribute to the process of keratinisation.  
32 Occassional mitotic indices (m) are seen in the



basal layer; (F) High magnification image of skin, showing greater detail of dermis (dm), epidermis (ed) and cornified layer (c). Again the stratum granulosum is visible (arrow). Scale bars: (A, B, C) 100  $\mu\text{m}$ ; (D, E) 25  $\mu\text{m}$ ; (F) 17.5  $\mu\text{m}$ .

Figure 7. Flow cytometry analysis of hESdF (left panel) and human foreskin fibroblasts (HFF, right panel) for the presence of CD31, CD44, CD71, CD90 and CD106. The bold (red) line represents the staining with the isotype control and the grey (green) line staining with specific antibodies.

**Figure 8.** Spontaneous differentiation of hES-NCL1 cells grown on hESdF and then in feeder-free conditions. hES-NCL1 differentiate into neuronal (A) and smooth muscle (B) cells demonstrating differentiation into cells of ectoderm and mesoderm, respectively. Green: cells stained with nestin antibody (A) and smooth muscle actin antibody (B). Red: cell-nuclei stained with propidium iodide. (A) shows small areas of red and green staining dispersed across the cells in a check-like pattern. (B) shows all cells stained green. Scale bars: 100  $\mu\text{m}$  (A) and 50  $\mu\text{m}$  (B).

## **Examples**

### **Material and Methods**

**Culture of embryos.** Two day old human embryos, produced by *in vitro* fertilization (IVF) for

1 clinical purposes, were donated by individuals  
2 after informed consent and after Human  
3 Fertilisation and Embryology Authority (HFEA, UK)  
4 approval. Until Day 3 (IVF = Day 0), 11 embryos  
5 were cultured in G1 medium and transferred to G2.3  
6 medium (both G1 & G2.3 from Vitrolife, Kungsbacka,  
7 Sweden) until day 6. Day 6 recovered blastocysts  
8 were cultured in Dulbecco's modified Eagle's medium  
9 (DMEM, Invitrogen, Paisley, Scotland) supplemented  
10 with 15% (v/v) Glasgow medium conditioned by  
11 Buffalo rat liver cells which has been used  
12 successfully for the long-term culture of bovine  
13 embryos, termed G-BRLC media (Stojkovic et al.,  
14 1995, Biol Reprod 53:1500-1507). On Day 8 ICMs  
15 were isolated by immunosurgery as previously  
16 described (Reubinoff et al., 2001, Hum Reprod  
17 10:2187-2194).

18  
19 **Cell-number analysis.** We investigated whether our  
20 three-step embryo culture supported development of  
21 Day 8 blastocysts and whether these blastocysts  
22 possess more ICM cells than Day 6 blastocysts.  
23 Eleven isolated ICMs from Day 6 blastocysts (5  
24 blastocysts and 6 expanded blastocysts) and 13 ICMs  
25 from Day 8 blastocysts (7 expanded and 6 hatching  
26 or hatched blastocysts) were analysed using 1.5  
27 µg/ml 4'-6-diamidino-2-phenylindole (DAPI, Sigma,  
28 St. Louis, MO) labelling as previously described  
29 (Spanos et al., 2000, Biol Reprod 63:1413-1420).

30

31 **Derivation of hES cells.** Initially, isolated ICMs  
32 were cultured on γ-irradiated MEFs monolayer

1 (75.000 cell/cm<sup>2</sup>) and DMEM supplemented with 10%  
2 (v/v) Hyclone defined fetal calf serum (FCS,  
3 Hyclone, Logan, UT) for 10 days. After 17 days, the  
4 hES cell colony was mechanically dispersed into  
5 several small clumps which were cultured on a fresh  
6 MEF layer with ES medium containing Knockout-DMEM  
7 (Invitrogen), 100 µM β-mercaptoethanol (Sigma), 1  
8 mM L-glutamine (Invitrogen), 100 mM non-essential  
9 amino acids, 10% serum replacement (SR,  
10 Invitrogen), 1% penicillin-streptomycin .  
11 (Invitrogen) and 4 ng/ml bFGF (Invitrogen). ES  
12 medium was changed daily. Human embryonic stem  
13 cells were passaged by incubation in 1 mg/ml  
14 collagenase IV (Invitrogen) for 5-8 minutes at 37°C  
15 or mechanically dissociated and then removed to  
16 freshly prepared MEF or hES cells-derived feeders.

17  
18 **Recovery of hES cell-derived fibroblasts.** Once a  
19 stable stem cell line was established, hES cells  
20 were transferred into feeder-free T-25 flasks  
21 (Iwaki, Asahi, Japan), using DMEM supplemented with  
22 10% FCS at 37°C in a 5% CO<sub>2</sub> atmosphere. After one  
23 week the stem cell derived fibroblast-like cells  
24 were transferred into T-75 flasks (Iwaki) and  
25 cultured for a further 3 days to produce a  
26 confluent primary monolayer of hES cells-derived  
27 fibroblasts.

28  
29 **Immunocytochemical analysis of hES cells and hES**  
30 **cells-derived fibroblasts.** Live staining was  
31 performed by adding primary antibodies (TRA1-60 and  
32 TRA1-81, a kind gift from Prof. P. Andrews

1 (University of Sheffield, UK) (but also available  
2 commercially from Chemicon); SSEA-4, SSEA-4 (MC-  
3 813-70) from Developmental Studies Hybridoma Bank,  
4 DSHB, Iowa City, IA; GCTM-2 and TG343, both a kind  
5 gift from Dr. M. Pera (Monash Institute of  
6 Reproduction and Development, Clayton, Australia);  
7 anti-fibroblast surface protein, AFSP from Sigma)  
8 to hES cells and hES cells-derived fibroblasts for  
9 20 minutes at 37°C. The primary antibodies were  
10 used at the following dilutions: TRA-1-60 - 1:10;  
11 TRA1-81 - 1:10; SSEA-3 - 1:4; SSEA-4 - 1:5  
12 (Henderson et al., 2002, Stem Cells 20:239-337);  
13 GCTM-2 - 1:2; AFSP - 1:50 (Ronnov-Jessen, 1992,  
14 Histochem Cytochem 40:475-486). TG343 at 1:2  
15 (Cooper et al., 2002, J Anat 200:259-265) was used  
16 to label cells grown on MEF feeder cells. The  
17 samples were gently washed three times with ES  
18 medium before being incubated with the 1:100  
19 secondary antibodies (anti mouse IgG and anti mouse  
20 IgM, both Sigma) conjugated to fluorescein  
21 isothiocyanate (FITC) at 37°C for 20 minutes. The  
22 samples were again washed three times with ES  
23 medium and subjected to fluorescence microscopy.  
24 For the Oct4 immunostaining hES cells were fixed in  
25 3.7% formaldehyde BDH, Coventry, UK for 20 minutes  
26 at room temperature followed by incubation in 3%  
27 hydrogen peroxide for 10 minutes. The hES cells  
28 were permeabilised with 0.2 % Triton x100 (Sigma)  
29 diluted in 4% sheep serum (Sigma) for 30 minutes at  
30 37°C. The ES colonies were incubated with the  
31 primary antibodies (Oct4 from Santa Cruz  
32 Biotechnologies, Heidelberg, Germany, final

1 concentration 10 µg/ml for 30 minutes at room  
2 temperature. The ES colonies were washed twice  
3 with PBS for 5 minutes and then incubated with the  
4 secondary antibody (rat anti mouse immunoglobulin  
5 (DAKO, Cambridgeshire, UK) used at 1:100 dilution)  
6 for 30 minutes at room temperature. After that,  
7 hES cells were washed again with PBS, incubated  
8 with ABC/HRP solution for 25 minutes at room  
9 temperature and washed again with PBS. The  
10 detection was carried out by incubation with DAB  
11 peroxidase (Enzo Life Sciences, NY) solution at  
12 room temperature for 1 minute. Final washes were  
13 done with distilled water. The bright field and  
14 fluorescent images were obtained using a Zeiss  
15 microscope and the AxioVision software (Carl Zeiss,  
16 Jena, Germany).

17  
18 **Comparison of hES cells-derived fibroblasts with**  
19 **human foreskin fibroblasts.** To identify the nature  
20 of feeder cells, hESdF were compared with human  
21 foreskin fibroblasts (HFF; ATCC, Teddington, UK)  
22 using flow-cytometry analysis. Briefly, hESdF were  
23 harvested using 0.05% Trypsin/0.53M EDTA  
24 (Invitrogen, Paisley, Scotland) and suspended in  
25 staining buffer (PBS +5% FCS) at concentration  $10^6$   
26 cells/ml. Hundred µl of the cell suspension was  
27 stained with 0.2 µg of CD31 (PECAM-1), CD71  
28 (Transferrin receptor), CD90 (Thy-1), and CD106  
29 (VCAM-1) antibodies (all available from BD  
30 Biosciences, Oxford, UK) at 4°C for 20 minutes.  
31 Three washes in staining buffer were carried out  
32 before staining with secondary antibody, goat anti-

1 mouse Ig-FITC (Sigma, Dorset, UK) used at 1:512  
2 dilution at 4°C for 20 minutes. Cells were washed  
3 again three times and resuspended in staining  
4 buffer before being analysed with FACS Calibur (BD)  
5 using the CellQuest software. 10,000 events were  
6 acquired for each sample and propidium iodide  
7 staining (1 µg/ml) was used to distinguish live  
8 from dead cells.

9  
10 **Karyotype analysis of hES cells and hES cells-**  
11 **derived fibroblasts.** The karyotype of hES cells  
12 and hES cells-derived fibroblasts was determined by  
13 standard G-banding procedure. A suitable protocol  
14 is available at:  
15 [http://www.slh.wisc.edu/cytogenetics/Protocols/Stai](http://www.slh.wisc.edu/cytogenetics/Protocols/Staining/G-Banding.html)  
16 [ning/G-Banding.html](http://www.slh.wisc.edu/cytogenetics/Protocols/Staining/G-Banding.html)

17  
18 **Reverse Transcription (RT)-PCR analysis.** The  
19 reverse transcription was carried out using the  
20 cells to cDNA II kit (Ambion, Huntingdon, UK)  
21 according to manufacturer's instructions. In  
22 brief, hES cells were submerged in 100 µl of ice-  
23 cold cell lysis buffer and lysed by incubation at  
24 75°C for 10 minutes. Genomic DNA was degraded by  
25 incubation with DNase I for 15 minutes at 37°C. RNA  
26 was reverse transcribed using M-MLV reverse  
27 transcriptase and random hexamers following  
28 manufacturer's instructions. PCR reactions were  
29 carried out using the following primers (Seq ID Nos  
30 1 to 12):

31  
32 OCT4 (F): 5'-GAAGGTATTCAGCCAAAC-3'; (SEQ ID No. 1)

31

1 OCT4 (R) : 5' - CTTAATCCAAAAACCCTGG-3' ; (SEQ ID No. 2)  
2 REX1 (F) : 5' -GCGTACGCAAATTAAAGTCCAGA-3' ; (SEQ ID No.  
3 3)  
4 REX1 (R) : 5' -CAGCATCCTAAACAGCTCGCAGAAT-3' ; (SEQ ID  
5 No. 4)  
6 NANOG (F) : 5' -GATCGGGCCCGCCACCATGAGTGTGGATCCAGCTTG-3' ;  
7 (SEQ ID No. 5)  
8 NANOG (R) : 5' -GATCGAGCTCCATCTTCACACGTCTTCAGGTTG-3' ;  
9 (SEQ ID No. 6)  
10 FOXD3F : 5' -GGAGGGAGGGGGCAATGCAC-3' ; (SEQ ID No. 7)  
11 FOXD3R : 5' -CCCCGAGCTCGCCTACT-3' ; (SEQ ID No. 8)  
12 TERT (F) : 5' -CGGAAGAGTGTCTGGAGCAAGT-3' ; (SEQ ID No.  
13 9)  
14 TERT (R) : 5' -GAACAGTGCCTTCACCCTCGA-3' ; (SEQ ID No.  
15 10)  
16 GAPDH (F) : 5' -GTCAGTGGTGGACCTGACCT-3' ; (SEQ ID No.  
17 11)  
18 GAPDH (R) : 5' -CACCACCCTGTTGCTGTAGC-3' (SEQ ID No.  
19 12).

20

21 Note that (F) and (R) refer to the direction of the  
22 primers and designate forward and reverse direction  
23 respectively.

24

25 PCR products were run on 2% agarose gels and  
26 stained with ethidium bromide. Results were  
27 assessed on the presence or absence of the  
28 appropriate size PCR products. Reverse  
29 transcriptase negative controls were included to  
30 monitor genomic contamination.

31

1     **DNA Genotyping of hES cells and hES cells-derived**  
2     **fibroblasts.** Total genomic DNA was extracted from  
3     both hES cells and hES cells-derived fibroblasts.  
4     DNA from both samples was amplified with 11  
5     microsatellite markers: D3S1358, vWA, D16S539,  
6     D2S1338, Amelogenin, D8S1179, D21S11, D18S51,  
7     D19S433, TH01, and FGA (Chen Y et al., 2003, Cell  
8     Res. 2003 Aug;13(4):251-63. full paper available at  
9     [http://www.cell-research.com/20034/2003-116/2003-4-](http://www.cell-research.com/20034/2003-116/2003-4-05-ShengHZ.htm)  
10    05-ShengHZ.htm) and analysed on an ABI 377 sequence  
11    detector using Genotype software (Applied  
12    Biosystems, Foster City, CA).

13  
14    **Growth of hES cells on hESdF.** HES-NCL1 cells were  
15    grown on  $\gamma$ -irradiated hESdF monolayer (75.000  
16    cells/cm<sup>2</sup>) in ES medium containing Knockout-DMEM  
17    (Invitrogen), 100  $\mu$ M  $\beta$ -mercaptoethanol (Sigma), 1  
18    mM L-glutamine (Invitrogen), 100 mM non-essential  
19    amino acids, 10% serum replacement (SR,  
20    Invitrogen), 1% penicillin-streptomycin  
21    (Invitrogen) and 4 ng/ml bFGF (Invitrogen). ES  
22    medium was changed daily. HES cells were passaged  
23    every 4-5 days by incubation in 1 mg/ml collagenase  
24    IV (Invitrogen) for 5-8 minutes at 37°C or  
25    mechanically dissociated and then removed to plates  
26    with freshly prepared hESdF.

27  
28    **Recovery of hESdF-conditioned medium.** Mitotically  
29    inactivated HESdF were cultured in T-25 flask with  
30    addition of ES medium for 10 days. hESdF-  
31    conditioned medium was collected every day and then  
32    frozen at -80°C.



1     **Growth of hES cells in feeder-free system using**  
2     **hESdF-conditioned medium.** hES cells were passaged  
3     and then removed to plates precoated with Matrigel  
4     (BD, Bedford, MA) (Xu et al., 2001, Nat Biotechnol  
5     19:971-974). ES media conditioned by hESdF was  
6     changed every 48 hours.

7  
8     **Cryopreservation of hES cells and hESdF.** To see  
9     whether frozen-thawed hESdF still support  
10    undifferentiated growth of cryopreserved hES cells,  
11    hESdF were frozen at -80°C using FCS supplemented  
12    with 10% (v/v) dimethyl sulfoxide (Sigma). Clumps  
13    of hES cells were frozen or vitrified using  
14    protocol as previously described (see Reubinoﬀ et  
15    al., 2001, Hum Reprod 10:2187-2194). Mitotic  
16    inactivation by using mitomycin C could  
17    alternatively be used.

18  
19    **Tumor formation in severe combined immunodeficient**  
20    **(SCID) mice (Stefan).** Ten to fifteen clumps with  
21    approximately 3000 hES cells in total were injected  
22    in kidney capsule, subcutaneously in flank or in  
23    the testis. After 21-90 days, mice were  
24    sacrificed, tissues were dissected, fixed in Bouins  
25    overnight, processed and sectioned according to  
26    standard procedures and counterstained with either  
27    haematoxylin and eosin or Weigerts stain. Sections  
28    were examined using bright field light microscopy  
29    and photographed as appropriate.

30

1 All procedures involving mice were carried out in  
2 accordance with institution guidelines and  
3 institution permission.

4

5 **Statistical analysis.** Cell numbers of Day 6 and Day  
6 8 ICMs were compared using Wilcoxon rank-sum test.  
7 The data are presented as mean  $\pm$  standard  
8 deviation.

9

10 **In vitro differentiation of hES cells.** Colonies of  
11 hES-NCL1 passage 21 were grown in feeder-free  
12 conditions in ES medium. After 5 to 14 days  
13 spontaneous differentiation was observed and  
14 differentiated cells were passaged and cultured  
15 under same conditions. Cells were fixed in 4%  
16 paraformaldehyde in PBS (Sigma) for 30 minutes and  
17 then permeabilised for additional 10 minutes with  
18 0.1% Triton X (Sigma). The blocking step was 30  
19 minutes with 2% FCS in PBS. Cells were incubated  
20 with antibody against nestin (1:200; Chemicon) or  
21 human alpha smooth muscle actin (1:50; Abcam,  
22 Cambridge, UK) for additional 2 hours. Each  
23 antibody was detected using corresponding secondary  
24 antibodies conjugated to FITC. The nuclei of cells  
25 were stained using propidium iodide for 5 minutes.

26

## 27 **Results**

28 Traditionally early blastocysts (Day 6) have been  
29 used for the derivation of human ES cell line. We  
30 developed a three - step culture system (see  
31 Materials and Methods) which supports successfully  
32 the development of late (Day 8) blastocysts.

1 Analysis of cell numbers of ICMS revealed that Day  
2 8 blastocysts possess significantly ( $P < 0.01$ ) more  
3 ICM cells than Day 6 blastocysts ( $51.3 \pm 9.6$  vs.  
4  $36.8 \pm 11.9$ , respectively). In view of this result  
5 we used day 8 blastocysts to derive human ES cell  
6 lines. Of the 11 Day 2 donated embryos, 7 (63.6%)  
7 blastocysts developed to Day 6. All 7 of these  
8 blastocysts expanded or hatched on Day 8 after  
9 transfer to G-BRLC medium. After isolation of ICMS  
10 by immunosurgery, 3 primary hES cell colonies  
11 showed visible outgrowth and one stable hES cell  
12 line (ICL-NCL1) was successfully derived (Figs. 1C-  
13 E).

14  
15 When the hES cells were cultured in the absence of  
16 feeder cells they spontaneously differentiated into  
17 fibroblast-like cells, ie. long, flat cells with  
18 elongated, condensed nucleus. We confirmed that  
19 the differentiated cells were fibroblasts by  
20 staining with a specific antibody to fibroblast  
21 surface protein (AFSP) (Fig. 2C and D). Karyotyping  
22 of the hES cells and hES cells-derived fibroblasts  
23 revealed that both samples are normal female ( $46 +$   
24  $XX$ , Figs. 2E and F). Microsatellite analysis  
25 revealed that the hES cells and hES cells-derived  
26 fibroblasts are indistinguishable from each other  
27 and should be considered as autogenic (see Fig. 2G,  
28 2H). We now have several batches of fresh and  
29 frozen/thawed serially expanded hES cells-derived  
30 fibroblasts which support hES cell culture even  
31 after the twelfth passage but they are optimal  
32 between second and eighth passages. Flow-cytometry

1 (Fig. 7) revealed that very few cells showed  
2 expression of mesenchymal cell specific markers  
3 CD106 (V-CAM1) and CD71 (transferring receptor) and  
4 none expressed the endothelial specific cell marker  
5 CD31 (PECAM-1). On the contrary, 94% and 82% of the  
6 hESdF cells were stained with the CD44 and CD90  
7 (THY-1) antibodies, respectively. Both antibodies  
8 were also presented in human foreskin fibroblasts  
9 (HFF; Fig. 7).

10

11 The hES-NCL1 line has been cultured on hES cell  
12 derived fibroblasts (hESdF) for over 35 passages  
13 and on Matrigel with hESdF conditioned medium for  
14 13 passages. We found that hES cell colonies grown  
15 on hES cell derived fibroblasts were dense, compact  
16 and suitable for mechanical passaging with typical  
17 morphology of hES cells (Fig. 4). Characterisation  
18 studies demonstrated that hES cells cultured on hES  
19 cells-derived fibroblasts or Matrigel with addition  
20 of hESdF-conditioned medium expressed specific  
21 surface markers: GTCM2, TRA1-60 and SSEA4, and  
22 (Fig. 4A-H) and were positive for the expression of  
23 *OCT-4*, *NANOG*, *FOXD3*, *REX-1* and *TERT* by RT-PCR (Fig.  
24 5A). Expression of TG343 was also found in hES  
25 cells grown on mouse feeder cells, and whilst not  
26 tested in the hESdF grown cells would be expected  
27 to be present. The fibroblast-like cells also  
28 expressed the telomerase reverse transcriptase  
29 (*TERT*) and *REX1* in early passages but none of the  
30 other ES cell specific markers. Human ES cells  
31 grafted into SCID mice consistently developed into  
32 teratomas demonstrating the pluripotency of hES-

1 NCL1 cells grown on hESdF. Teratomas were primarily  
2 restricted to the site of injection and their  
3 histological examination revealed advanced  
4 differentiation of structures representative of all  
5 three embryonic germ layers, including cartilage,  
6 skin, muscle, primitive neuroectoderm, neural  
7 ganglia, secretory epithelia and connective tissues  
8 (Fig. 6). When hES-NCL1 cells were cultured in  
9 absence of feeders and Matrigel, spontaneous  
10 differentiation into neuronal (Fig. 8A) and smooth  
11 muscle (Fig. 8B) cells was observed.

12

13